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18-MC Acts in the Medial Habenula and Interpeduncular Nucleus to Attenuate Dopamine Sensitization to Morphine in the Nucleus Accumbens

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KEY WORDS opioids; mesolimbic pathway; drug addiction; habenulo-interpeduncular pathway

ABSTRACT 18-Methoxycoronaridine (18-MC), a novel iboga alkaloid congener, is a potential treatment for drug addiction. 18-MC has been shown to decrease self-administration of drugs (e.g., morphine, methamphetamine, nicotine) and attenuate opioid withdrawal in rats. In previous studies, systemic pretreatment with 18-MC abolished the sensitized increase in accumbens dopamine levels induced by chronic morphine administration. In vitro studies have shown that 18-MC is a potent antagonist of $\alpha 3\beta 4$ nicotinic receptors, and $\alpha 3\beta 4$ antagonism is believed to be the primary mechanism responsible for 18-MC's effects on drug self-administration and possibly on morphine-induced changes in mesolimbic dopamine. While there are very low densities of $\alpha 3\beta 4$ nicotinic receptors in the mesolimbic pathway, these receptors are prominently localized in the medial habenula (MHb) and in the interpeduncular nucleus (IPN). These nuclei and the habenulo-interpeduncular pathway connecting them are believed to function as part of an alternate reward pathway modulating the dopaminergic mesolimbic pathway known to be involved in drug addiction. In the present study, to determine if 18-MC acts in the MHb or in the IPN, the effects of local infusion of 18-MC into these brain areas were assessed on mesolimbic dopamine responses to acute and repeated morphine treatment. Administration of 18-MC (10 μ g) into either the IPN or MHb blocked the sensitized dopamine response to repeated morphine in the nucleus accumbens; 18-MC had no effect on the dopamine response to acute morphine. The results suggest that 18-MC acts in the habenulo-interpeduncular pathway to modulate the effects of repeated morphine in the dopaminergic mesolimbic system. **Synapse 61:547–560, 2007.** © 2007 Wiley-Liss, Inc.

INTRODUCTION

The activation of the mesolimbic system induced by morphine and other opioids is well-characterized and is believed to be primarily mediated by μ -opioid receptors located on GABA-ergic interneurons in the ventral tegmental area (Johnson and North, 1992). The repeated administration of morphine can induce the sensitization of dopaminergic neurotransmission in the mesolimbic system; multiple neuronal adaptations that accompany this phenomenon are thought to be linked to persistent craving and relapse in human addicts (Robinson and Berridge, 1993). Thus, therapeutic agents that could reverse the hypersensitivity of the mesolimbic system might be efficacious in treating opioid addiction.

18-MC, a synthetic congener of ibogaine, is currently being investigated as a potential therapeutic agent for opioid addiction. In animal studies, systemic pretreatment with 18-MC has been shown to reduce the i.v. self-administration of morphine and other drugs (Glick et al., 1996, 2000) and alleviate several signs of acute opioid withdrawal in rats (Rho and Glick, 1998). Along with these effects, 18-MC has been shown to interfere with morphine-induced dopamine

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release in the mesolimbic system. Thus, a single i.p. injection of 18-MC enhanced the effect of acute morphine on dopamine levels in the nucleus accumbens while it abolished the sensitized increase in accumbens dopamine levels in rats repeatedly exposed to morphine (Maisonneuve and Glick, 1999; Szumlinski et al., 2000).

Functional studies have shown that 18-MC is a potent antagonist of $\alpha 3\beta 4$ nicotinic receptors ($IC_{50} = 0.75 \mu M$) (Glick et al., 2002), while being inactive at $\alpha 4\beta 2$ nicotinic receptors, the most prevalent nicotinic receptor subtype in the brain (Gotti et al., 2006). The ability of 18-MC to antagonize $\alpha 3\beta 4$ nicotinic receptors is believed to be the primary mechanism responsible for attenuation of opioid self-administration and expression of somatic signs of opioid withdrawal in rats (Maisonneuve and Glick, 2003). Consistent with this hypothesis, combinations of low doses of nonselective $\alpha 3\beta 4$ antagonists were also effective in reducing morphine self-administration (Maisonneuve and Glick, 2003) and in attenuating several signs of acute opioid withdrawal in rats (Taraschenko et al., 2005).

In the central nervous system, $\alpha 3\beta 4$ nicotinic receptors are predominantly located in the medial habenula and interpeduncular nucleus, while very low densities of them are present in the nuclei of the mesolimbic pathway (Picciotto, 2003; Wonnacott, 1997). The projections from the medial habenula to the interpeduncular nucleus are predominantly cholinergic and pass in the core of the fasciculus retroflexus as the habenulo-interpeduncular pathway (Sutherland, 1982). The habenulo-interpeduncular pathway has both direct and indirect connections to both the ventral tegmental area and the nucleus accumbens (Sutherland, 1982). Functional interactions between the mesolimbic and the habenulo-interpeduncular pathways may be dependent on those connections (Nishikawa et al., 1986). The habenulo-interpeduncular and the mesolimbic pathways may jointly mediate rewarding and neurochemical effects of addictive drugs (Ellison, 1994). Consistent with this hypothesis, direct administration of 18-MC into the medial habenula or interpeduncular nucleus attenuated self-administration of morphine in rats (Glick et al., 2006). In the present study, in an attempt to assess the role of the habenulo-interpeduncular pathway in 18-MC's modulation of morphine's mesolimbic effects, we locally administered 18-MC into the MHb and the IPN while conducting microdialysis in the nucleus accumbens of freely-moving rats.

MATERIALS AND METHODS

Animals

Experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (1996) and were approved by the Institutional

Animal Care and Use Committee of Albany Medical College. Naïve female Sprague-Dawley rats (Taconic, Germantown, NY), weighing 250–285 g, were housed individually in polyurethane cages in a colony room and maintained on a normal 12:12-h light/dark cycle (light on 7 a.m., light off at 7 p.m.). Food and water were provided ad libitum.

Drugs

18-Methoxycoronaridine hydrochloride (Albany Molecular Research, Albany, NY) was dissolved in 50% DMSO solution (vehicle), pH = 5.0, and injected intracranially at the concentrations of 5, 10, or 20 $\mu g/\mu l$ (volume = 1 μl). Morphine sulfate (Research Biochemicals, Natick, MA) was dissolved in saline and injected intraperitoneally at 5 mg/kg for acute studies or 20 mg/kg for repeated administration studies. Drug solutions were made fresh for each experiment.

Stereotaxic brain cannulation surgery

The surgery was performed according to the previously described protocol (Maisonneuve and Glick, 1999). Specifically, for the acute studies, the rats were implanted with one microdialysis guide cannula (CMA Microdialysis, North Chelmsford, MA) over the nucleus accumbens and one microinjection guide cannula (22-gauge) (Plastics One, Roanoke, VA) over the ipsilateral medial habenula or interpeduncular nucleus. For the repeated administration studies, the animals were implanted with two microdialysis guide cannulae bilaterally over the nucleus accumbens and one microinjection guide cannula over the medial habenula or interpeduncular nucleus. The sides for the microinjection guide were alternated from rat to rat. Coordinates in millimeter were chosen according to Paxinos and Watson (1986) such that when a dialysis probe was inserted, the tip was located in the nucleus accumbens [in mm, AP = +1.6; ML = ± 3.0 ; DV = -8.3 using a 14° angle] and when an injector was lowered the tip was located in the medial habenula (in mm, AP = -4.2 ; ML = ± 2.9 ; DV = -6.0 using a 24° angle), or interpeduncular nucleus (in mm, AP = -6.3 ; ML = ± 3.0 ; DV = -10.4 using a 24° angle). Rats were monitored daily for proper recovery and allowed to recover for 4–5 days before any testing was performed.

In vivo microdialysis studies

Acute studies

The microdialysis experiment was conducted in an environmentally controlled room, which was maintained at a normal day/night cycle (light on 7 a.m., light off at 7 p.m.). On the afternoon before the microdialysis, the 2 mm dialysis probes were calibrated for dopamine, DOPAC, and HVA prior to use in vivo in

order to avoid using probes with in vitro recoveries less than 15% (Glick et al., 1994). The rats were placed in the dialysis chamber and a dialysis probe was inserted through the guide cannula on one side of the brain. The probe was continuously perfused at a flow rate of 1 μ l/min with artificial cerebrospinal fluid (146 mM NaCl, 2.7 mM KCL, 1.2 mM CaCl₂, 1.0 mM MgCl₂). The collection of brain perfusates began the following morning. The samples were collected in tubes containing 2 μ l of 1.1 N perchloric acid solution (containing 50 mg/L Na₂EDTA and 50 mg/L sodium metabisulfite). After collection of six 20-min baseline samples, rats received an injection of 18-MC into the MHb or IPN, which was immediately followed by an i.p. injection of morphine or saline. The collection of dialysate samples was then continued for 3 h. The samples were analyzed for dopamine, DOPAC, and HVA with high performance liquid chromatography (HPLC). Upon completion of a microdialysis experiment, the animals were sacrificed and their brains were processed for verification of probe and injector placements.

Repeated morphine administration studies

The experiment was conducted using a previously described "within-subject" paradigm (Szumlinski et al., 2000) such that each rat was dialyzed twice, 7 days apart, from opposite sides of the brain. Each animal served as its own control in the statistical analysis; such a design reduces intersubject variability and provides a more reliable assessment of dopamine sensitization in morphine-treated rats. The preparation of rats for the first microdialysis session was identical to that described for acute studies. On the day of the experiment, after collection of six baseline samples, rats were injected intraperitoneally with morphine (20 mg/kg). The effect of morphine was subsequently monitored for 3 h. Upon completion of a microdialysis session, the probe was removed and the rats were returned to their home cages in the colony room. For the following 4 days, rats were taken out of the colony room every 24 h for daily injections of morphine (20 mg/kg, i.p.). The treatment was administered in the rats' home cages while in the microdialysis room; rats were maintained in the same environment for 3 h afterwards and then subsequently returned to the colony room. This repeated morphine treatment procedure, previously utilized in our laboratory (Szumlinski et al., 2000), allows animals to develop morphine-context associations that promotes the expression of dopamine sensitization on the test day (Stewart, 1992). Following ~48 h of withdrawal, animals were prepared for the second microdialysis session and probes were inserted into the contralateral nucleus accumbens (and ipsilateral to the intracerebral injection side). Upon collection of baseline

samples, (precisely after 72 h of withdrawal), rats received intracerebral (MHb or IPN) injections of 18-MC or vehicle followed immediately by a challenge injection of morphine (20 mg/kg, i.p.). The effect of morphine was monitored for the following 3 h. Upon completion of this session animals were euthanized, and their brains were saved for histological analyses of probe and injector sites. The samples from each dialysis session were analyzed immediately for dopamine and metabolites with HPLC.

Intracerebral drug administration

Immediately before intracerebral injection, dummy stylet was removed from the microinjection guide cannula and 18-MC (or vehicle) was delivered into the MHb or IPN by means of a microsyringe (Hamilton, Reno, NV) in a volume of 1 μ l over a 1-min period via the 26-gauge injector (Plastics One, Roanoke, VA). The injector was kept in place for an additional minute after the solution was administered.

Verification of the probe and injector sites

Upon completion of experiments rats were euthanized in a CO₂ chamber. Brains were rapidly removed and frozen at -80°C until thin-sectioned in cryostat (40 μ m). Only the dialysates from animals with probes located within the boundaries of the nucleus accumbens and injectors located within the boundaries of the MHb/IPN were accepted for analysis (Figs. 1 and 5).

High performance liquid chromatography

The high performance liquid chromatography system with electrochemical detection (HPLC-ED) was comprised of an ESA 540 autosampler (ESA, North Chelmsford, MA), an ESA solvent delivery unit, an ESA column (MD-150/RP-C18; diameter = 3.0 μ m), and an ESA Coulochem II electrochemical detector with an ESA 5020 guard cell and an ESA 5014B analytical cell. The glassy carbon working electrode was set at a potential of 300 mV with respect of the reference electrode. The MD-TM mobile phase (ESA, North Chelmsford, MA) was comprised of 75 mM sodium dihydrogen phosphate monohydrate, 1.7 mM 1-octanesulfonic acid sodium salt, 100 μ l/L triethylamine, 25 μ M EDTA in 10% acetonitrile (pH = 3.0); it was pumped at a flow rate of 0.530 ml/min. Chromatograms were processed using Agilent Technologies Chem Station Plus software (Agilent Technologies, Wilmington, DE).

Statistical analysis

The basal levels of dopamine and metabolites expressed as pm/10 μ l were analyzed with repeated measures analysis of variance (ANOVA) with dose of

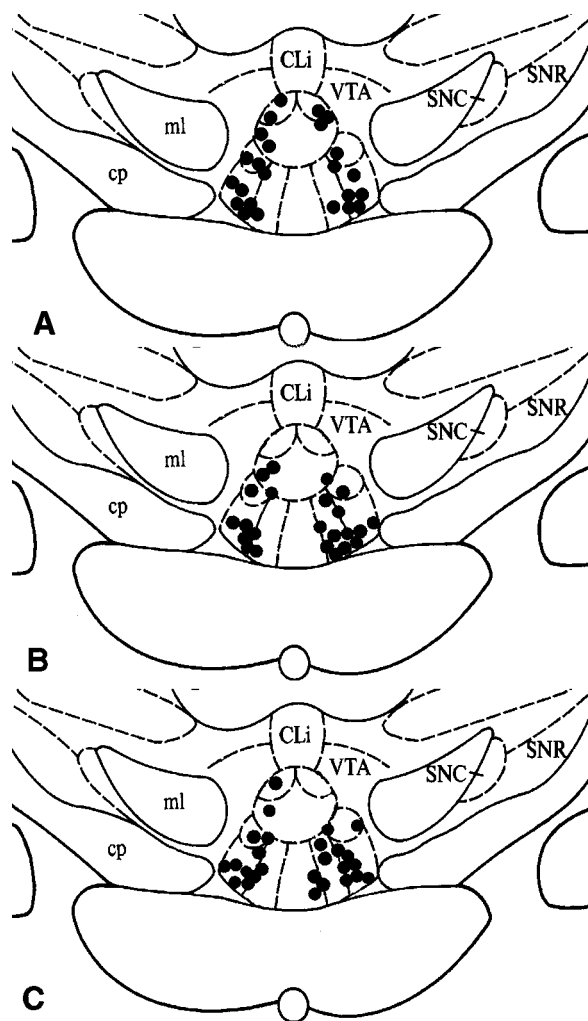


Fig. 1. Localization of microinjection cannulae placements in the interpeduncular nucleus of saline-treated animals (A), $n = 24$; animals treated with acute morphine (B), $n = 23$ and animals treated repeated morphine (C), $n = 27$. CLi, caudal linear raphe nucleus; SNC, substantia nigra compacta; SNR, substantia nigra reticular; VTA, ventral tegmental area; ml, medial lemniscus; cp, cerebral peduncle.

18-MC (0, 5, 10, and 20 μg) as independent factor and time as the repeated measures variable. If the basal concentration of extracellular dopamine or metabolites were not significantly different among the treatment groups, the levels of dopamine and metabolites were expressed as percents of the appropriate baseline means and were used as such for all subsequent analyses. For the acute studies the data were analyzed with repeated measures ANOVA with dose of 18-MC as independent factor and time as the repeated measures variable. For the repeated morphine administration studies, the data for each treatment group for both microdialysis sessions were analyzed with repeated measures ANOVA with session (Week 1 and Week 2) and time as the two repeated

measure factors. The ANOVAs were followed by post-hoc comparison tests (Fisher LSD) when appropriate.

RESULTS

Effects of 18-MC in the interpeduncular nucleus

Microinjection sites of animals accepted for analysis were located from 6.04 to 6.72 mm posterior to bregma (Paxinos and Watson, 1986) and were positioned within the boundaries of the IPN (Fig. 1). Microdialysis probe placements were positioned within the shell of the nucleus accumbens, with an area outlined as medial two-thirds of a distance between the midline and the anterior commissure (not shown). The coordinates ranged from 1.2 to 1.7 mm anterior to bregma (Paxinos and Watson, 1986).

Effects of 18-MC on dopamine and metabolite release in the nucleus accumbens of saline-treated rats (Fig. 2)

The average basal levels of extracellular dopamine, DOPAC, and HVA in the nucleus accumbens were 0.014 ± 0.003 , 10.143 ± 1.272 and 5.533 ± 0.853 pmol/10 μl , respectively (mean $\pm$ SEM, $n = 24$). Analysis of basal extracellular concentrations of dopamine and metabolites revealed no significant difference among pretreatment groups (for dopamine, Group effect: $F_{3, 20} = 0.21$, $P > 0.89$; for DOPAC, Group effect: $F_{3, 20} = 1.37$, $P > 0.28$; for HVA, Group effect: $F_{3, 20} = 1.80$, $P > 0.18$). Administration of vehicle into the IPN did not alter basal release of dopamine or its metabolites in the nucleus accumbens (for dopamine, Time effect: $F_{14, 70} = 0.95$, $P > 0.52$; for DOPAC, Time effect: $F_{14, 70} = 1.14$, $P > 0.34$; for HVA, Time effect: $F_{14, 70} = 1.43$, $P > 0.16$). The infusion of 18-MC into the IPN did not alter dopaminergic neurotransmission in the nucleus accumbens (for dopamine, Group effect: $F_{3, 20} = 1.06$, $P > 0.39$, Group $\times$ Time interaction: $F_{42, 280} = 0.90$, $P > 0.65$; for DOPAC, Group effect: $F_{3, 20} = 0.36$, $P > 0.78$, Group $\times$ Time interaction: $F_{42, 280} = 0.97$, $P > 0.53$; for HVA, Group effect: $F_{3, 20} = 1.09$, $P > 0.38$, Group $\times$ Time interaction: $F_{42, 280} = 1.08$, $P > 0.36$).

Effects of 18-MC on mesolimbic dopaminergic responses to acute morphine (Fig. 3)

The average basal level of extracellular dopamine in the nucleus accumbens was 0.010 ± 0.001 pmol/10 μl (mean $\pm$ SEM, $n = 23$). There were no differences in basal concentrations of dopamine among pretreatment groups (Group effect: $F_{3, 19} = 0.37$, $P > 0.78$). The average basal levels of extracellular DOPAC and HVA were 10.319 ± 0.812 pmol/10 μl and 6.595 ± 0.721 pmol/10 μl , respectively. Basal concentrations of dopamine metabolites were not different among the four pretreatment groups (for DOPAC, Group effect:

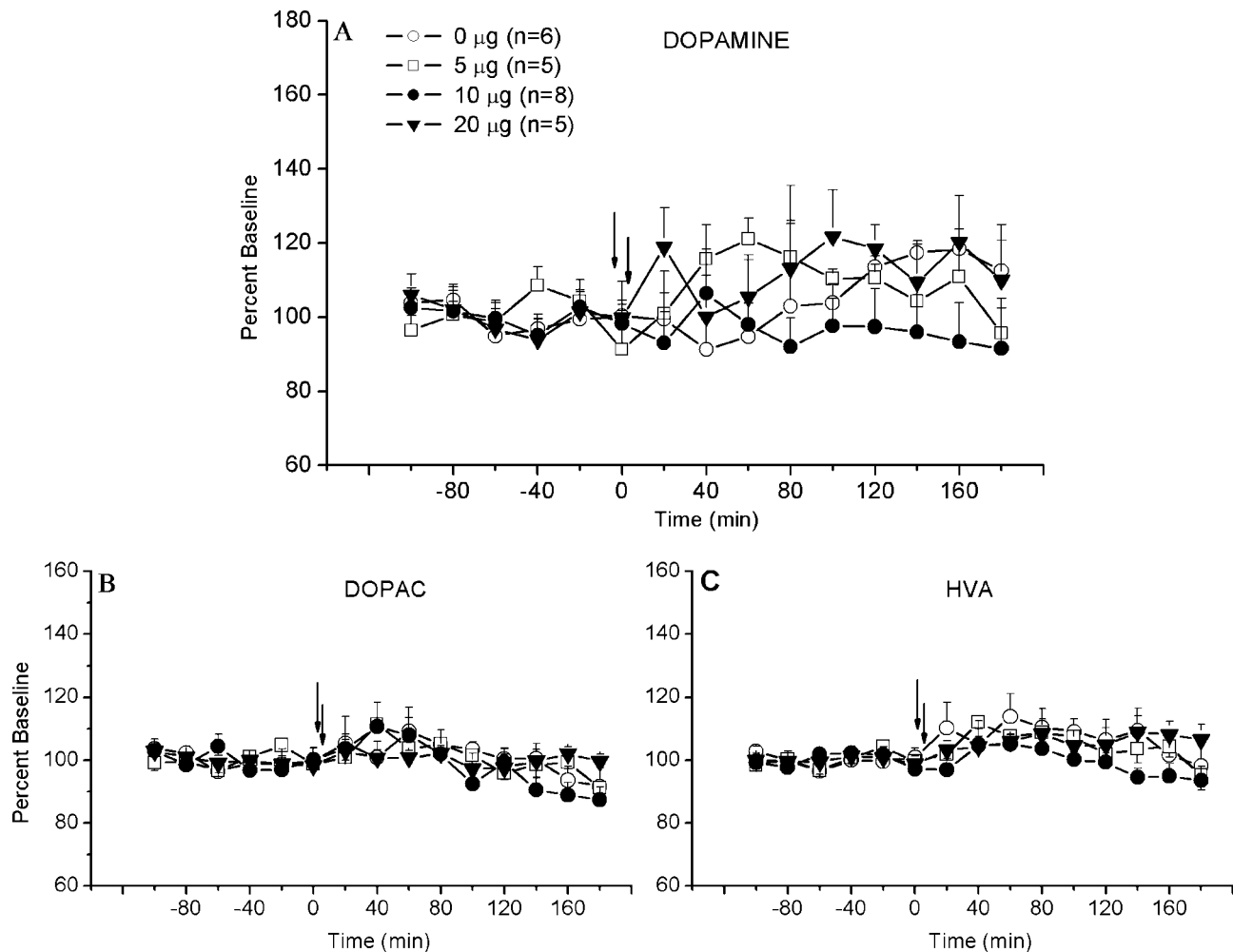


Fig. 2. **A–C:** Time-course of extracellular dopamine (**A**), DOPAC (**B**) and HVA(**C**) (mean $\pm$ SEM) in the nucleus accumbens of saline-treated rats infused with 18-MC into the interpeduncular nucleus. Arrows indicate pretreatment with 18-MC (5, 10, 20 μ g) followed by saline (i.p.).

$F_{3, 19} = 0.14$, $P > 0.93$ and for HVA, Group effect: $F_{3, 19} = 0.17$, $P > 0.92$).

Consistent with previous reports (Maisonneuve and Glick, 1999; Maisonneuve et al., 2001), i.p. injection of morphine (5 mg/kg) in vehicle-pretreated rats increased extracellular levels of dopamine in the nucleus accumbens, with a maximum of 41% at 60 min after treatment (Time effect: $F_{14, 56} = 5.33$, $P < 0.00001$; Fig. 3A). In the same group of animals, morphine also increased extracellular levels of DOPAC and HVA in the nucleus accumbens (for DOPAC, Time effect: $F_{14, 56} = 17.62$, $P < 0.00001$ and for HVA, Time effect: $F_{14, 56} = 31.67$, $P < 0.00001$; Figs. 3B–3C); the DOPAC was highest (42%) at 100 min after injection, while HVA reached its peak (53% increase) at 140 min after injection.

As shown in Figure 3, infusion of 18-MC into the interpeduncular nucleus did not alter morphine-induced increases in dopamine or its metabolites in the nucleus accumbens (for dopamine, Group effect:

$F_{3, 19} = 0.05$, $P > 0.99$, Group $\times$ Time interaction: $F_{42, 266} = 0.66$, $P > 0.95$; for DOPAC, Group effect: $F_{3, 19} = 0.31$, $P > 0.82$, Group $\times$ Time interaction: $F_{42, 266} = 0.70$, $P > 0.92$; for HVA, Group effect: $F_{3, 19} = 0.02$, $P = 1.00$, Group $\times$ Time interaction: $F_{42, 266} = 0.39$, $P = 1.00$).

Effects of 18-MC on mesolimbic dopaminergic responses to repeated morphine (Fig. 4)

The average basal levels of extracellular dopamine in the nucleus accumbens prior to the first morphine injection (session 1) and after repeated morphine administration (session 2) were 0.015 ± 0.003 pmol/10 μ l and 0.012 ± 0.002 pmol/10 μ l, respectively (mean $\pm$ SEM, $n = 26$). The basal levels of dopamine assessed for each microdialysis sessions were not significantly different among the pretreatment groups (for week 1, Group effect: $F_{3, 22} = 0.42$, $P > 0.74$; for week 2, Group effect: $F_{3, 22} = 0.86$, $P > 0.48$). For all

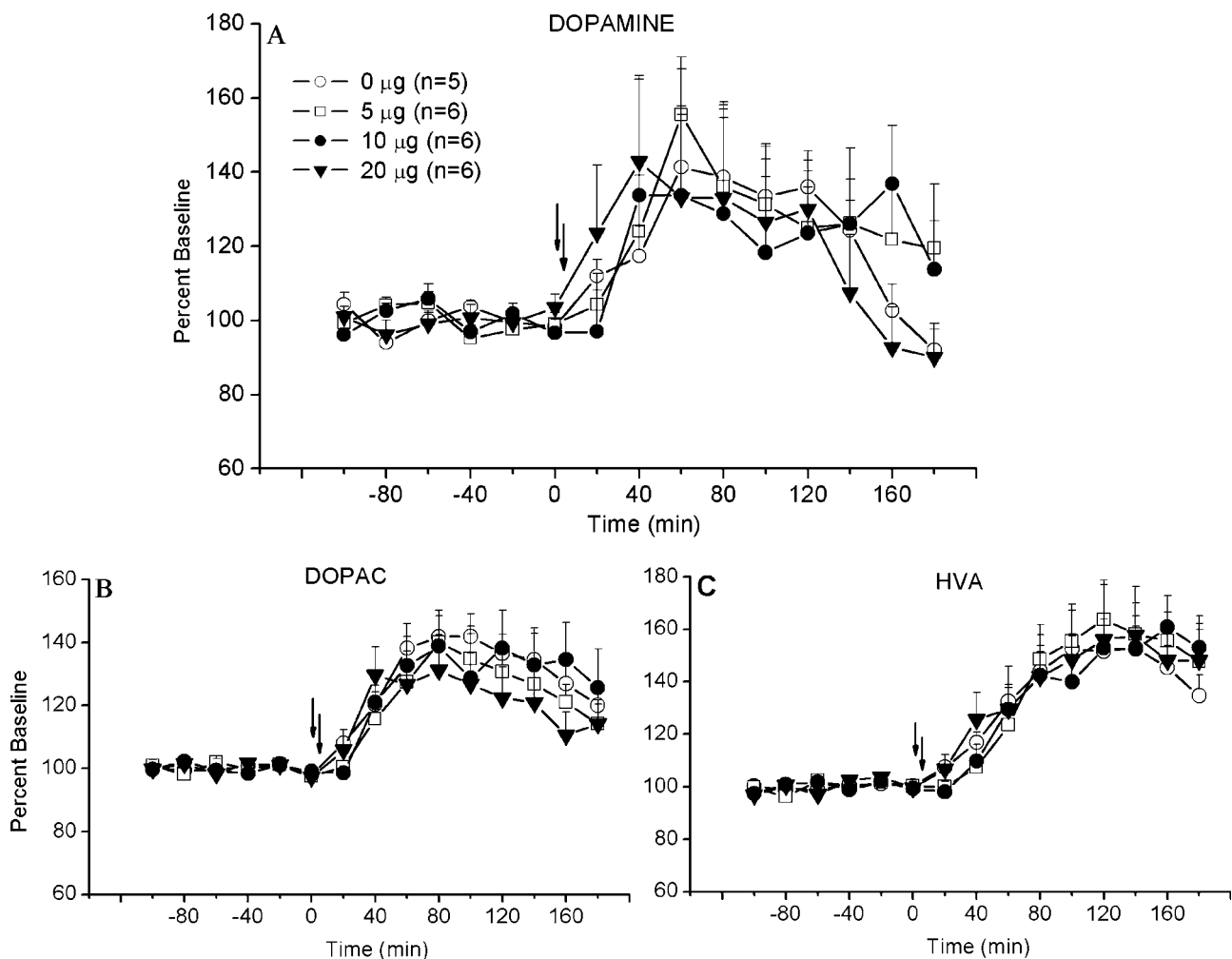


Fig. 3. A–C: Time-course of extracellular dopamine (A), DOPAC (B) and HVA (C) (mean $\pm$ SEM) in the nucleus accumbens of morphine-treated rats infused with 18-MC into the interpeduncular nucleus. Arrows indicate injection of 18-MC (5, 10, 20 μ g or vehicle) followed by morphine (5 mg/kg i.p.).

pretreatment groups, repeated morphine administration had no effect on basal extracellular levels of dopamine in the nucleus accumbens (Week effect: $F_{1, 25} = 0.64$, $P > 0.43$). Measured in the same assay, the average basal levels of extracellular DOPAC and HVA for the week 1 session were 9.889 ± 0.913 and 6.579 ± 0.687 pmol/10 μ l, respectively. The mean basal levels of metabolites for the Week 2 session were 8.365 ± 0.690 pmol/10 μ l (for DOPAC) and 4.212 ± 0.395 pmol/10 μ l (for HVA). The levels of metabolites assessed for each dialysis session were not significantly different among pretreatment groups (for DOPAC in week 1, Group effect: $F_{3, 22} = 0.67$, $P > 0.58$; for Week 2, Group effect: $F_{3, 22} = 1.14$, $P > 0.35$; for HVA in Week 1, Group effect: $F_{3, 22} = 0.99$, $P > 0.42$; for Week 2, Group effect: $F_{3, 22} = 2.35$, $P > 0.10$). The basal levels of metabolites were significantly reduced after repeated morphine administration (for DOPAC, Week effect: $F_{1, 25} = 10.29$, $P < 0.004$; for HVA, Week effect: $F_{1, 25} = 9.60$, $P < 0.005$).

Consistent with previous reports (Maisonneuve et al., 2001; Szumlinski et al., 2000), the first injection of morphine (20 mg/kg i.p.) in naïve rats produced significant increases in dopamine levels in the nucleus accumbens in all treatment groups (Group effect: $F_{3, 22} = 0.04$, $P > 0.99$, Time effect: $F_{14, 308} = 10.53$, $P < 0.00001$; Group $\times$ Time interaction: $F_{42, 308} = 0.31$, $P = 1.00$, Fig. 4A). Vehicle-pretreated animals displayed dopamine sensitization to repeated morphine administration during the second dialysis session as compared to the first session (Week $\times$ Time interaction: $F_{14, 98} = 1.78$, $P < 0.05$). Sensitization of the dopamine response was apparent at 40–100 and 160–180 min after challenge injection of morphine (post-hoc tests). In contrast, dopamine sensitization was not observed in the 18-MC pretreated groups (for 18-MC 5 μ g, Week effect: $F_{1, 7} = 1.90$, $P > 0.21$, Week $\times$ Time interaction: $F_{14, 98} = 1.43$, $P > 0.15$; for 18-MC 10 μ g, Week effect: $F_{1, 5} = 0.13$, $P > 0.73$, Week $\times$ Time interaction: $F_{14, 70} = 0.41$,

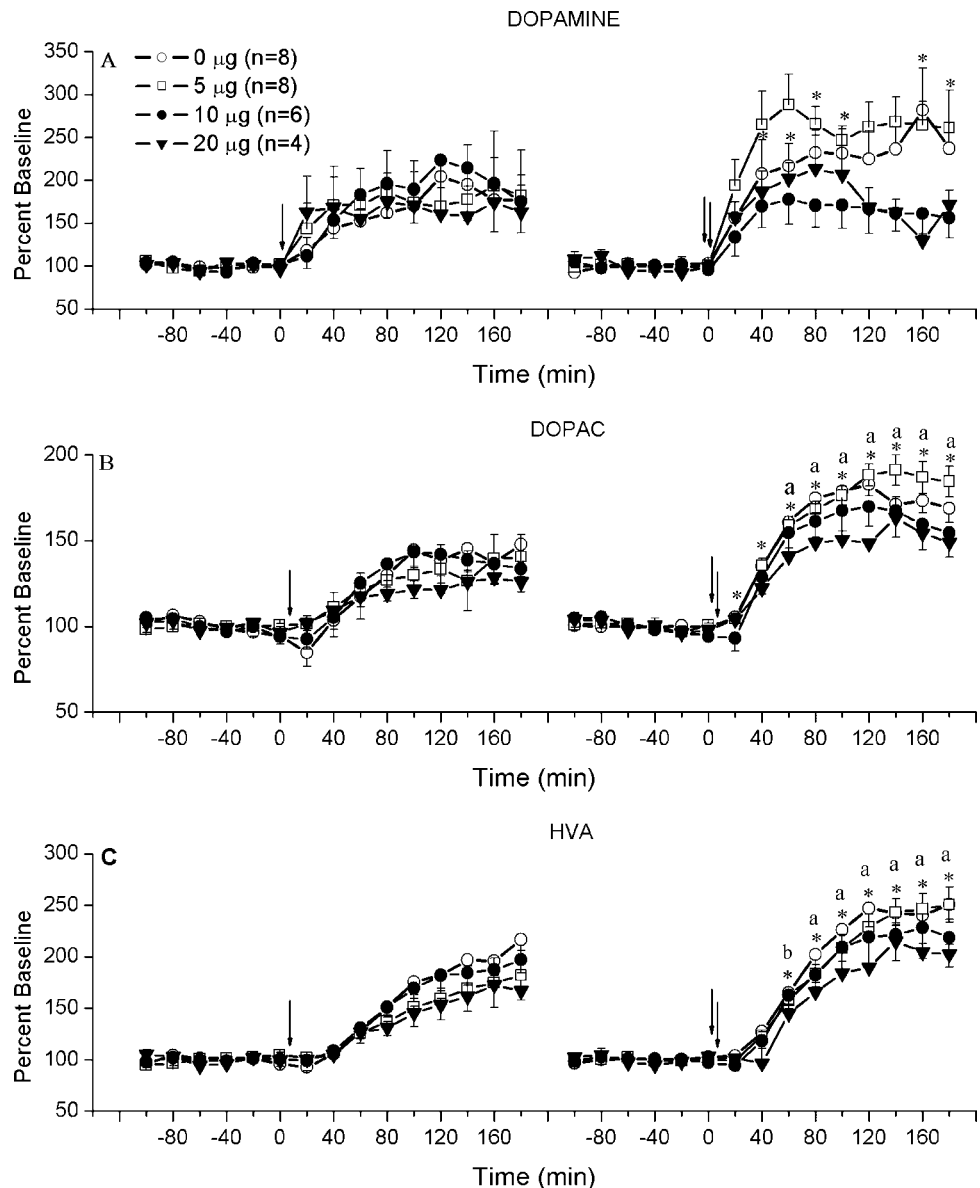


Fig. 4. A–C: Time-course of extracellular dopamine (A), DOPAC (B) and HVA (C) (mean $\pm$ SEM) in the nucleus accumbens during Week 1–Week 2 of rats repeatedly treated with morphine. Arrow in Week 1 session indicates injection morphine (20 mg/kg i.p.); arrows in Week 2 session indicate infusions of 18-MC into the interpeduncular nucleus (5, 10, 20 μ g or vehicle) followed by morphine (20 mg/kg i.p.). Sensitization in the treatment groups is indicated as follows: (for vehicle; *a* for 5 and 20 μ g; *b* for 5 μ g and *c* for 20 μ g ($P < 0.05$, LSD test).

$P > 0.97$; for 18-MC 20 μ g, Week effect: $F_{1, 3} = 1.35$, $P > 0.33$, Week $\times$ Time interaction: $F_{14, 42} = 1.00$, $P > 0.47$). This lack of sensitization was most apparent for the 10 μ g dose of 18-MC (see Fig. 4A).

As shown in Figures 4B–C, an acute dose of morphine in all naïve rats produced increases in dopamine metabolism, raising extracellular levels of DOPAC and HVA (for DOPAC, Group effect: $F_{3, 22} = 0.13$, $P > 0.94$, Time effect: $F_{14, 308} = 22.89$, $P < 0.00001$; Group $\times$ Time interaction: $F_{42, 308} = 0.70$, $P > 0.92$; for HVA, Group effect: $F_{3, 22} = 0.58$, $P > 0.64$, Time effect: $F_{14, 308} = 65.73$, $P < 0.00001$; Group $\times$ Time interaction: $F_{42, 308} = 0.90$, $P > 0.65$). Vehicle-pretreated animals displayed sensitization of DOPAC levels during the second dialysis session as compared to the first session (Week $\times$ Time interaction: $F_{14, 98}$

$= 7.14$, $P < 0.00001$); sensitization was evident from 20 to 180 min after morphine. Similarly, sensitization of DOPAC levels was also observed in groups pretreated with 5 and 20 μ g of 18-MC (for 18-MC 5 μ g, Week $\times$ Time interaction: $F_{14, 98} = 7.01$, $P < 0.00001$; for 18-MC 20 μ g, Week $\times$ Time interaction: $F_{14, 42} = 4.60$, $P < 0.00006$); the sensitized responses were apparent from 60 to 180 after test injection of morphine (Fig. 4B). In contrast, repeated morphine administration did not produce sensitization in the group pretreated with 10 μ g of 18-MC (Week effect: $F_{1, 5} = 2.00$, $P > 0.22$, Week $\times$ Time interaction: $F_{14, 70} = 1.60$, $P > 0.10$).

Statistical analysis of HVA responses in vehicle-pretreated animals revealed sensitization of HVA levels during the second dialysis session as compared to the

first session (Week $\times$ Time interaction: $F_{14, 98} = 6.55$, $P < 0.00001$). The sensitization was apparent from 60 to 180 min after injection of morphine (Fig. 4C). Sensitization of HVA responses were also present in groups pretreated with 5 and 20 μg of 18-MC but not in the group that received 10 μg of 18-MC (for 18-MC 5 μg , Week $\times$ Time interaction: $F_{14, 98} = 5.49$, $P < 0.00001$; for 18-MC 10 μg , Week effect: $F_{1, 5} = 1.24$, $P > 0.32$, Week $\times$ Time interaction: $F_{14, 70} = 1.28$, $P > 0.24$; for 18-MC 20 μg , Week $\times$ Time interaction: $F_{14, 42} = 3.34$, $P < 0.00001$). As shown in Figure 4C, sensitization was apparent at 60 and 80 min after test injection for 5 and 20 μg , respectively and persisted until the end of the dialysis session. Thus, pretreatment with 18-MC (10 μg) reduced sensitization of dopamine and dopamine metabolites in rats previously exposed to morphine.

Effects of 18-MC in the medial habenula

Local infusions of 18-MC were confined within the boundaries of the medial habenula from 3.80 to 4.30 mm posterior to bregma (Fig. 5) (Paxinos and Watson, 1986). Similar to the experiments described above, the membranes of the microdialysis probes were located within the shell of the nucleus accumbens (not shown).

Effects of 18-MC on dopamine and metabolite release in the nucleus accumbens of saline-treated rats (Fig. 6)

The average basal levels of extracellular dopamine, DOPAC, and HVA in the nucleus accumbens were 0.012 ± 0.003 , 10.886 ± 0.954 , and 5.767 ± 0.499 pmol/10 μl , respectively (mean $\pm$ SEM, $n = 23$). There were no differences in basal concentrations of dopamine and metabolites among pretreatment groups (for dopamine, Group effect: $F_{3, 19} = 0.47$, $P > 0.71$; for DOPAC, Group effect: $F_{3, 19} = 0.33$, $P > 0.80$; for HVA, Group effect: $F_{3, 19} = 0.34$, $P > 0.80$). Local infusion of vehicle into the medial habenula did not affect basal dopaminergic neurotransmission in the nucleus accumbens (for dopamine, Time effect: $F_{14, 70} = 0.96$, $P > 0.50$; for DOPAC, Time effect: $F_{14, 70} = 0.96$, $P > 0.50$; for HVA, Time effect: $F_{14, 70} = 1.24$, $P > 0.27$). Similarly, the infusion of 18-MC into the medial habenula did not produce an effect on basal levels of dopamine or of its metabolites (for dopamine, Group effect: $F_{3, 19} = 0.12$, $P > 0.95$, Group $\times$ Time interaction: $F_{42, 266} = 0.77$, $P > 0.85$; for DOPAC, Group effect: $F_{3, 19} = 0.06$, $P > 0.98$, Group $\times$ Time interaction: $F_{42, 266} = 0.74$, $P > 0.88$; for HVA, Group effect: $F_{3, 19} = 1.15$, $P > 0.35$, Group $\times$ Time interaction: $F_{42, 266} = 1.14$, $P > 0.26$).

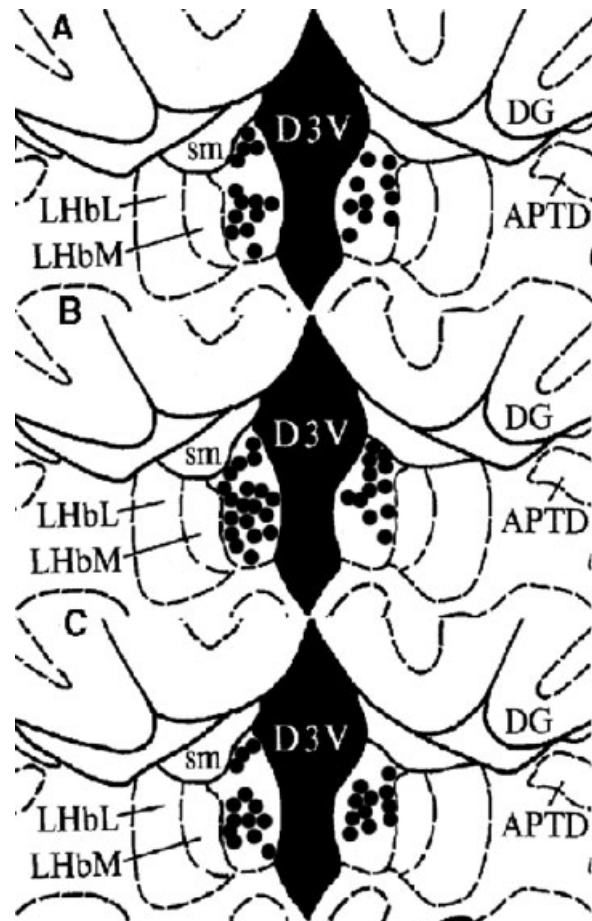


Fig. 5. Localization of microinjection cannulae placements in the medial habenula of saline-treated animals (A), $n = 23$; animals treated with acute morphine (B), $n = 32$, and animals treated repeated morphine (C), $n = 24$. D3V, dorsal third ventricle; LHbL, lateral habenular nucleus, lateral; LHbM, lateral habenular nucleus, medial; sm, stria medullaris; DG, dentate gyrus; APTD, anterior pretectal nucleus, dorsal.

Effects of 18-MC on mesolimbic dopaminergic responses to acute morphine (Fig. 7)

The average basal level of extracellular dopamine, DOPAC, and HVA in the nucleus accumbens were 0.013 ± 0.002 , 12.089 ± 0.820 , and 6.430 ± 0.376 pmol/10 μl , respectively (mean $\pm$ SEM, $n = 32$). Basal concentrations of dopamine metabolites were not different among the four pretreatment groups (for dopamine, Group effect: $F_{3, 28} = 0.27$, $P > 0.85$; for DOPAC, Group effect: $F_{3, 28} = 0.07$, $P > 0.97$; for HVA, Group effect: $F_{3, 28} = 1.04$, $P > 0.39$).

As expected (Maisonneuve and Glick, 1999; Maisonneuve et al., 2001), acute injection of morphine (5 mg/kg) in vehicle-pretreated rats increased extracellular levels of dopamine in the nucleus accumbens; the maximum increase of 53% was reached at 60 min after treatment (Time effect: $F_{14, 98} = 3.91$, $P < 0.00003$; Fig. 7A). The extracellular levels of

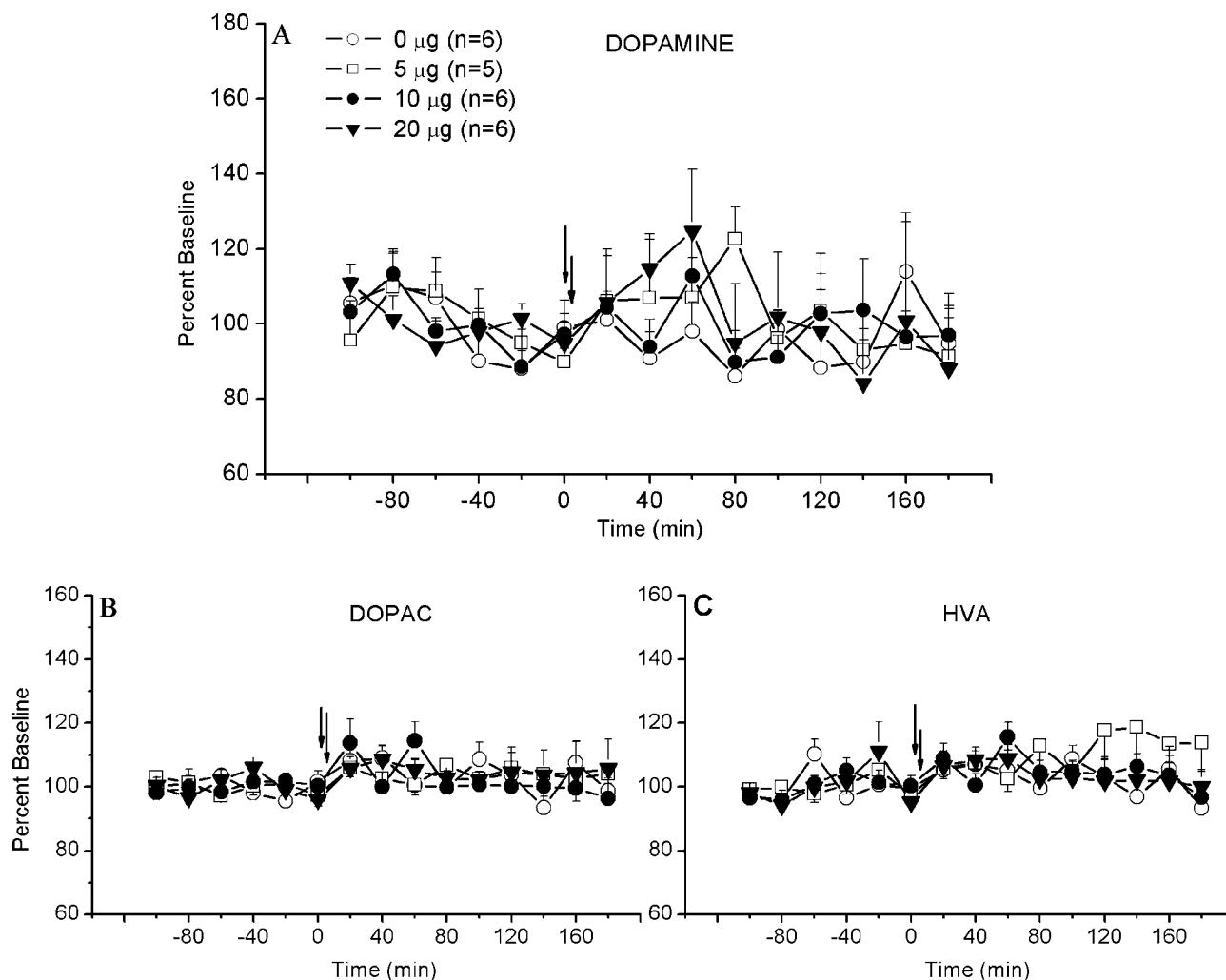


Fig. 6. **A–C:** Time-course of extracellular dopamine (**A**), DOPAC (**B**) and HVA (**C**) (mean $\pm$ SEM) in the nucleus accumbens of saline-treated rats infused with 18-MC into the medial habenula. Arrows indicate pretreatment with 18-MC (5, 10, 20 μ g) followed by saline (i.p.).

DOPAC and HVA were also increased in the same animals (for DOPAC, Time effect: $F_{14, 98} = 10.05$, $P < 0.00001$ and for HVA, Time effect: $F_{14, 98} = 17.17$, $P < 0.00001$). As shown in Figure 7, the maximums of 35% and 47% were achieved at 100 and 120 min after injection for DOPAC and HVA, respectively.

Analysis of data for all four pretreatment groups revealed no effects of local infusion of 18-MC into the medial habenula on morphine-induced responses of dopamine and its metabolites in the nucleus accumbens (for dopamine, Group effect: $F_{3, 28} = 0.63$, $P > 0.60$, Group $\times$ Time interaction: $F_{42, 392} = 0.73$, $P > 0.90$; for DOPAC, Group effect: $F_{3, 28} = 0.78$, $P > 0.52$, Group $\times$ Time interaction: $F_{42, 392} = 0.67$, $P > 0.94$; for HVA, Group effect: $F_{3, 28} = 1.35$, $P > 0.28$, Group $\times$ Time interaction: $F_{42, 392} = 1.05$, $P > 0.38$).

Effects of 18-MC on mesolimbic dopaminergic responses to repeated morphine (Fig. 8)

The average basal levels of extracellular dopamine in the nucleus accumbens in session 1 and session 2 of dialysis were 0.011 ± 0.002 and 0.011 ± 0.001 pmol/10 μ l, respectively (mean $\pm$ SEM, $n = 24$). Comparison of dopamine levels for each week of dialysis revealed no differences in basal concentration among pretreatment groups (for Week 1, Group effect: $F_{3, 20} = 1.52$, $P > 0.24$; for Week 2, Group effect: $F_{3, 20} = 0.85$, $P > 0.48$). Repeated administration of morphine did not affect basal concentrations of dopamine in the nucleus accumbens (Week effect: $F_{1, 23} = 0.03$, $P > 0.86$). In the same animals, the mean basal levels of DOPAC for each dialysis session were 16.199 ± 0.957 and 15.467 ± 0.982 pmol/10 μ l, respectively, while the levels of HVA were 6.808 ± 0.532 and $6.385 \pm$

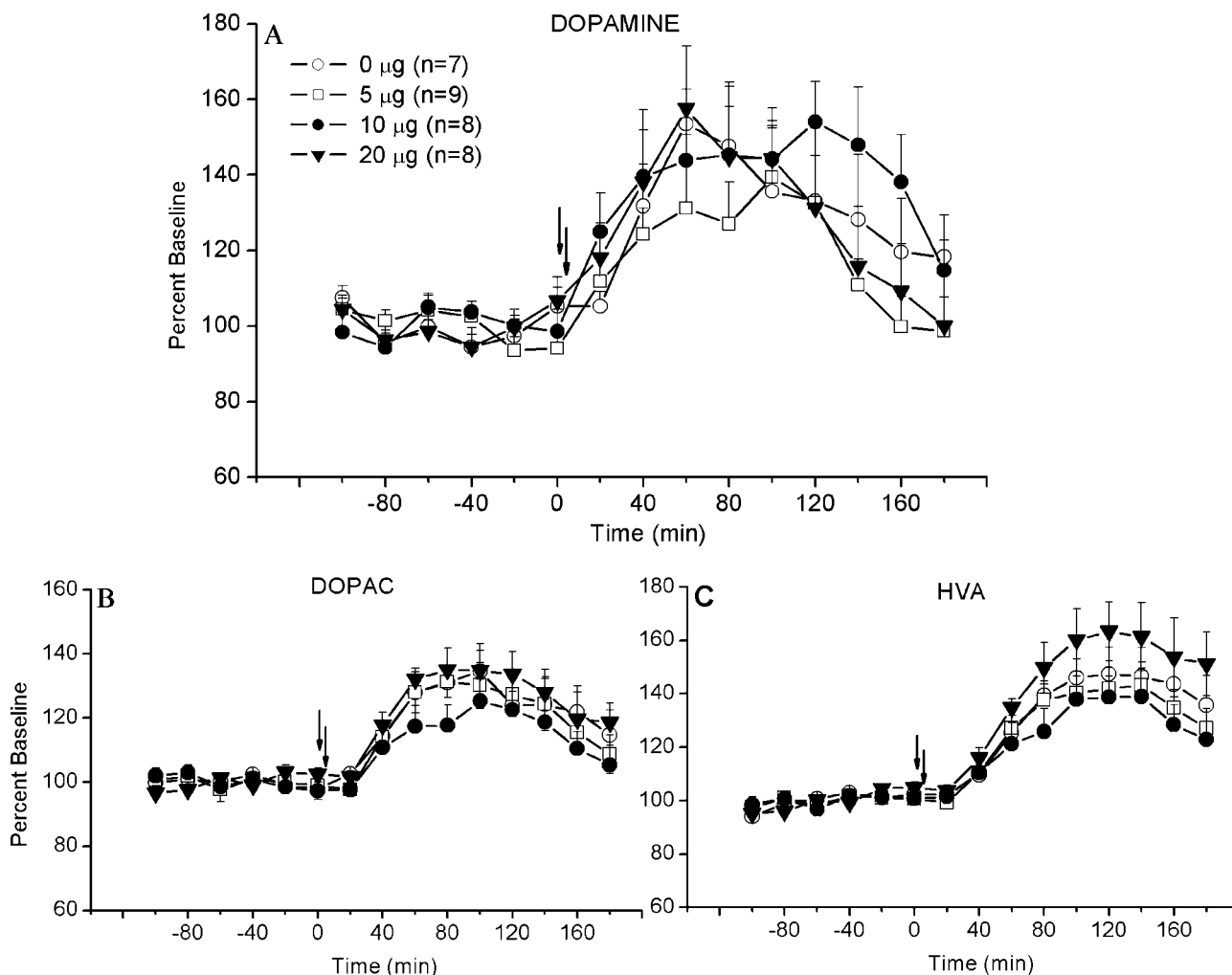


Fig. 7. A–C: Time-course of extracellular dopamine (A), DOPAC (B) and HVA (C) (mean $\pm$ SEM) in the nucleus accumbens of morphine-treated rats infused with 18-MC into the medial habenula. Arrows indicate injection of 18-MC (5, 10, 20 μ g or vehicle) followed by morphine (5 mg/kg i.p.).

0.401 pmol/10 μ l. There were no differences in basal metabolite levels among the pretreatment groups in either dialysis session (for DOPAC in week 1, Group effect: $F_{3, 20} = 1.98$, $P > 0.15$; in Week 2, Group effect: $F_{3, 20} = 2.30$, $P > 0.11$; for HVA in Week 1, Group effect: $F_{3, 20} = 1.25$, $P > 0.32$; in Week 2, Group effect: $F_{3, 20} = 2.68$, $P > 0.07$). The repeated administration of morphine did not alter levels of DOPAC or HVA (for DOPAC: Week effect: $F_{1, 23} = 0.72$, $P > 0.40$; for HVA: Week effect: $F_{1, 23} = 0.52$, $P > 0.48$).

As shown in Figure 8A, an acute injection of morphine (20 mg/kg i.p.) during the first microdialysis session produced significant increases in dopamine release in the nucleus accumbens of all treated rats (Group effect: $F_{3, 20} = 0.05$, $P > 0.98$, Time effect: $F_{14, 288} = 12.69$, $P < 0.00001$; Group $\times$ Time interaction: $F_{42, 280} = 0.27$, $P = 1.00$). Consistent with a previous study using the same repeated morphine administra-

tion protocol (Szumlinski et al., 2000), vehicle-pretreated animals displayed dopamine sensitization to repeated morphine administration during the second dialysis session as compared to the first session; this response was apparent from 60 to 120 min after morphine (Week Time interaction: $F_{14, 84} = 1.92$, $P < 0.04$; post-hoc tests). Sensitization also occurred in the group locally infused with 20 μ g of 18-MC but not with 5 or 10 μ g of 18-MC (for 18-MC 20 μ g, Week $\times$ Time interaction: $F_{14, 56} = 2.01$, $P < 0.03$; for 18-MC 5 μ g, Week effect: $F_{1, 4} = 2.05$, $P > 0.23$, Week $\times$ Time interaction: $F_{14, 56} = 0.45$, $P > 0.95$; for 18-MC 10 μ g, Week effect: $F_{1, 6} = 0.38$, $P > 0.56$, Week $\times$ Time interaction: $F_{14, 84} = 0.45$, $P > 0.95$). Sensitized dopamine responses in the group pretreated with 20 μ g of 18-MC were expressed from 80 to 140 min after morphine administration.

Extracellular levels of DOPAC and HVA were significantly increased in all groups of rats after the first

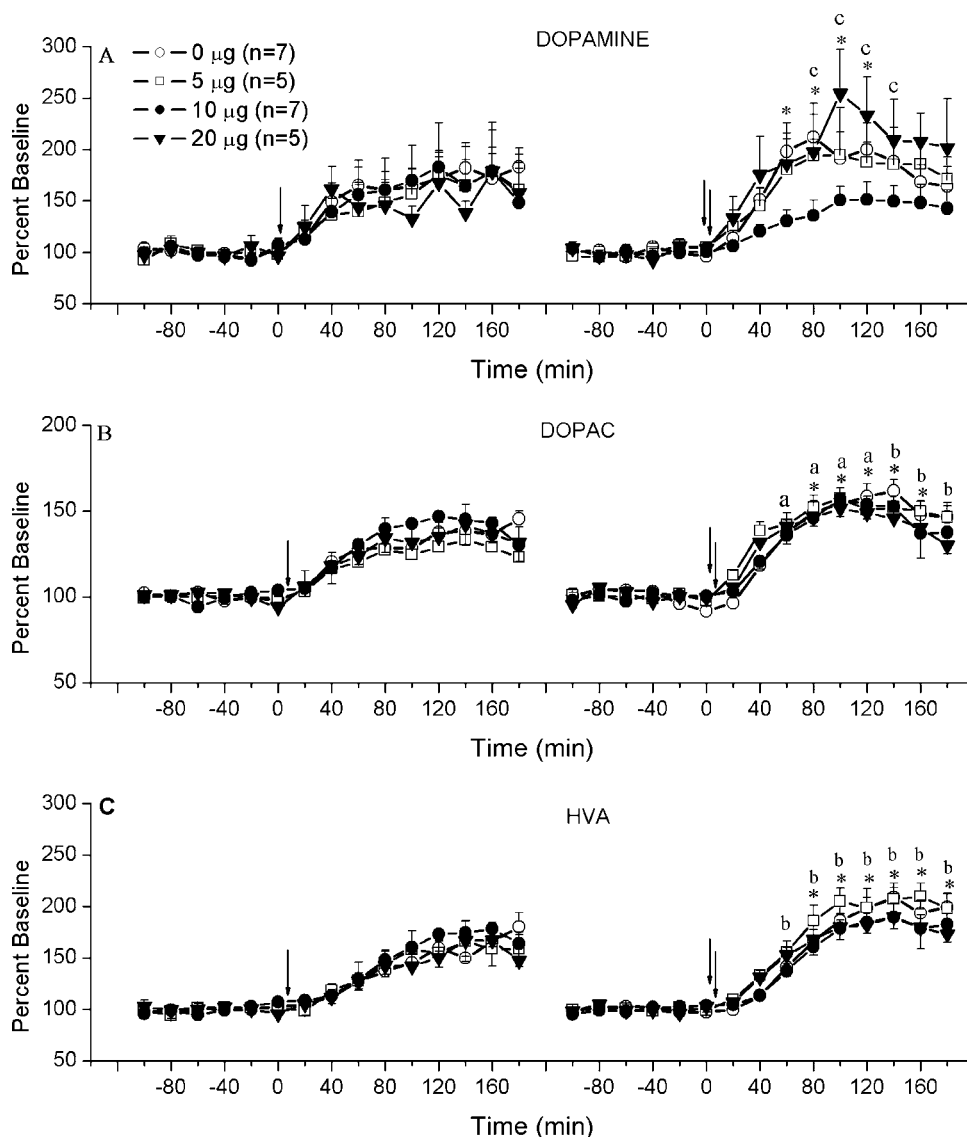


Fig. 8. A–C: Time-course of extracellular dopamine (A), DOPAC (B) and HVA (C) (mean $\pm$ SEM) in the nucleus accumbens during Week 1–Week 2 of rats repeatedly treated with morphine. Arrow in Week 1 session indicates injection morphine (20 mg/kg i.p.); arrows in Week 2 session indicate infusions of 18-MC into the medial habenula (5, 10, 20 μ g or vehicle) followed by morphine (20 mg/kg i.p.). Sensitization in the treatment groups is indicated as follows: (for vehicle; a for 5 and 20 μ g; b for 5 μ g and c for 20 μ g ($P < 0.05$, LSD test).

injection of morphine. (for DOPAC, Group effect: $F_{3, 20} = 0.25$, $P > 0.86$, Time effect: $F_{14, 322} = 40.44$, $P < 0.00001$; Group $\times$ Time interaction: $F_{42, 280} = 0.64$, $P > 0.96$; for HVA, Group effect: $F_{3, 20} = 0.13$, $P > 0.94$, Time effect: $F_{14, 322} = 44.98$, $P < 0.00001$; Group $\times$ Time interaction: $F_{42, 280} = 0.51$, $P > 0.99$). Analysis of DOPAC responses in vehicle-pretreated animals revealed sensitization in the second week as compared to the first week; sensitization was evident from 80 to 160 min after morphine injection (Week $\times$ Time interaction: $F_{14, 84} = 5.33$, $P < 0.00001$; post-hocs). Further analysis showed that sensitization of DOPAC levels also occurred in the groups receiving 5 and 20 μ g but not 10 μ g of 18-MC (for 18-MC 5 μ g, Week $\times$ Time interaction: $F_{14, 56} = 2.25$, $P < 0.02$; for 18-MC 20 μ g, Week $\times$ Time interaction: $F_{14, 56} = 2.15$, $P < 0.02$; for 18-MC 10 μ g, Week effect: $F_{1, 6} = 0.28$, $P > 0.62$, Week $\times$ Time interaction: $F_{14, 84} =$

0.40, $P > 0.99$). The sensitized responses in the former groups were apparent from 60 to 180 and from 60 to 120 min after test injection of morphine, respectively (Fig. 8B).

Analysis of HVA responses in vehicle-pretreated group showed sensitization of HVA levels during the second session as compared to the first session; these responses were significant from 80 to 180 min after morphine (Week $\times$ Time interaction: $F_{14, 84} = 7.64$, $P < 0.00001$; post-hocs). Further analysis revealed sensitization in the group pretreated with 5 μ g but not with 10 or 20 μ g (for 18-MC 5 μ g, Week $\times$ Time interaction: $F_{14, 56} = 4.33$, $P < 0.00004$; for 18-MC 10 μ g, Week effect: $F_{1, 6} = 0.28$, $P > 0.62$, Week $\times$ Time interaction: $F_{14, 84} = 0.41$, $P > 0.99$; for 18-MC 20 μ g, Week effect: $F_{1, 4} = 2.40$, $P > 0.20$, Week $\times$ Time interaction: $F_{14, 56} = 1.60$, $P > 0.11$). As shown in Figure 8C, the levels of HVA in the group locally

infused with 5 μ g of 18-MC remained sensitized from 60 min after morphine until the end of the dialysis session. These experiments showed that 18-MC (10 μ g), locally infused into the medial habenula, attenuated the expression of sensitization of dopamine release and metabolism in rats repeatedly treated with morphine.

DISCUSSION

The results of the present study suggest that the two nuclei of the habenulo-interpeduncular pathway are responsible for the previously described actions of systemic 18-MC on mesolimbic responses to repeated morphine (Szumlinski et al., 2000). As measured with in vivo microdialysis, attenuation of morphine-sensitized release of dopamine and its metabolites in the nucleus accumbens was achieved with infusion of 10 μ g of 18-MC into either the medial habenula or interpeduncular nucleus. The habenulo-interpeduncular pathway conveys primarily cholinergic fibers in the core of the fasciculus retroflexus from the medial habenula in the diencephalon to the interpeduncular nucleus in the midbrain (Sutherland, 1982). This pathway was described by Blander and Wise (1989) as a distinct system supporting self-stimulation reward (Blander and Wise, 1989). Subsequently, the habenulo-interpeduncular pathway was shown to be functionally interrelated with the mesolimbic pathway (Nishikawa et al., 1986). Specifically, self-stimulation of the habenula was facilitated by ipsilateral lesions of the medial forebrain bundle, while self-stimulation of the medial forebrain bundle was increased by ipsilateral lesions of the habenula (Sutherland, 1982). Tetrodotoxin-induced interruption of the fasciculus retroflexus increased dopamine synthesis and metabolism in homogenates of the nucleus accumbens (Nishikawa et al., 1986). Taken collectively, these studies suggested that the habenulo-interpeduncular and the mesolimbic pathways might jointly mediate rewarding effects of addictive drugs (Ellison, 1994). Consistent with this premise, 18-MC infused into the medial habenula or interpeduncular nucleus attenuated self-administration of morphine (Glick et al., 2006).

Interactions between the two pathways during opioid administration may involve changes in local dynamics of dopamine, acetylcholine and other neurotransmitters. Morphine, when administered acutely, enhances dopamine release in the nucleus accumbens by μ -receptor-mediated disinhibition of dopamine neurons in the ventral tegmental area (Johnson and North, 1992); δ -opioid receptors in the nucleus accumbens may also be involved (Borg and Taylor, 1997). Repeated administration of morphine induces sensitized dopamine responses, in part mediated by adaptations in glutamate and dopamine neurotransmis-

sion (Vanderschuren and Kalivas, 2000). In the habenulo-interpeduncular pathway, acute morphine alters release of acetylcholine in the interpeduncular nucleus and tolerance develops to this effect during repeated administration (Taraschenko et al., 2006); these changes may be mediated by opioid receptors in the medial habenula and interpeduncular nucleus (Atweh and Kuhar, 1977; Bot and Chahl, 1996). In the present study, during repeated morphine administration, the habenulo-interpeduncular pathway was shown to modulate the release of dopamine in the nucleus accumbens.

Functional interactions between the mesolimbic and the habenulo-interpeduncular pathways probably rely on multiple anatomical connections between the nuclei of the two pathways (Sutherland, 1982). For example, direct afferents from the medial habenula to the ventral tegmental area may alter the firing pattern of dopamine neurons; such an interaction may involve glutamate (Sutherland, 1982). On the other hand, the interpeduncular nucleus can influence the release of dopamine in the nucleus accumbens via sequential glutamatergic connections between the mediodorsal thalamus and the medial prefrontal cortex (Morley, 1986; Pennartz et al., 1994; Vertes, 2006).

Although the exact mechanism underlying the effects of 18-MC in the present study is not clear, it may involve the antagonism of $\alpha 3\beta 4$ nicotinic receptors densely expressed in the nuclei of the habenulo-interpeduncular pathway (Quick et al., 1999). In the medial habenula, $\alpha 3\beta 4$ receptors are located on acetylcholine- and glutamate-containing neurons and presumably participate in modulation of cholinergic inputs from the nucleus of the diagonal band of Broca and posterior septal area (McCormick and Prince, 1987; Quick et al., 1999). In the interpeduncular nucleus, $\alpha 3\beta 4$ nicotinic receptors are located on the terminals of the habenular afferents and on local GABAergic interneurons (Lena et al., 1993; Mulle et al., 1991). Together with muscarinic receptors, $\alpha 3\beta 4$ nicotinic receptors can modulate the effects of acetylcholine released from neuronal projections to the IPN from the basal forebrain, the brainstem and the medial habenula (Morley, 1986). For example, acetylcholine was shown to elicit either facilitatory or inhibitory modulation of synapses in the IPN, depending on participation of either nicotinic or muscarinic receptors, respectively (Girod and Role, 2001). Thus, if 18-MC locally infused into the IPN blocked nicotinic receptors, this might produce a switch to inhibitory muscarinic dominance. The projections from the IPN to the NAC would then be inhibited, and attenuation of dopamine release in the nucleus accumbens would occur. On the other hand, in the medial habenula, antagonism of $\alpha 3\beta 4$ nicotinic receptors could remove an excitatory influence of the habenula on the ventral tegmental area, also resulting in attenuation of sensi-

tized dopamine release in the nucleus accumbens. Although 18-MC has some activity at all opioid receptors, the low affinities make it unlikely that an opioid mechanism is involved in the above described effects (Glick et al., 2001).

In the present study the highest dose of 18-MC was not effective in reducing the sensitization of dopamine. Although no in vitro data exists to support this speculation, it is conceivable that at higher doses 18-MC binds to the other nicotinic receptors subtypes, such as heteropentameric $\beta 4$ - or $\beta 2$ -containing nicotinic receptors (e.g., $\alpha 3\beta 3\beta 4$ or $\alpha 2\beta 2$) located in the interpeduncular nucleus (Gotti et al., 2006). The antagonism of these receptors might produce effects that oppose those produced by antagonism of $\alpha 3\beta 4$ receptors. For example, 18-MC might prevent GABA release in the IPN, shown to be regulated by $\beta 2$ -containing nicotinic receptors in this nucleus (Gotti et al., 2006); this may oppose glutamatergic activation as proposed in the mechanism for the effect of the lower dose of 18-MC. A similar action of 18-MC at other receptors in the medial habenula (Gotti et al., 2006) is possible as well.

Because of the proximity of the VTA to the IPN, 18-MC could possibly diffuse into the VTA and act directly on the mesolimbic pathway; however this is unlikely to be the case. The VTA has few $\alpha 3\beta 4$ receptors and 18-MC has little or no affinity for $\alpha 4\beta 2$ receptors (Glick et al., 2002), the main nicotinic receptor subtype in the VTA (Klink et al., 2001). Moreover, 18-MC locally infused into the VTA had no effect on rates of morphine self-administration, while a substantial effect was produced by the same treatment administered into the IPN (Glick et al., 2006).

The present results suggest that a previously described effect of systemic 18-MC on mesolimbic dopamine release in morphine-naïve rats (Maisonneuve and Glick, 2003) was not mediated by its action on either the medial habenula or the interpeduncular nucleus alone. It is conceivable that in morphine-naïve animals, connections between the mesolimbic and the habenulo-interpeduncular pathways have little functional significance as compared to these pathways in animals sensitized to morphine. Alternatively, the additive action of 18-MC on both nuclei, rather than on a single nucleus, might be required to alter the acute effect of morphine on dopamine neurotransmission in the mesolimbic pathway.

Sensitization of the mesolimbic dopaminergic pathway in response to drugs of abuse is believed to be a neurochemical substrate for persistent drug-seeking behavior and relapse in human addicts (Robinson and Berridge, 1993). However, the contribution of other brain systems in some behavioral aspects of drug addiction cannot be dismissed. For example, persistent changes in glutamate neurotransmission in the medial prefrontal cortex induced by stimulant abuse

is thought to be responsible for compulsive drug-directed behavior in humans (Vanderschuren and Kalivas, 2000). In the dorsal striatum, phasic dopamine release in addicted humans was linked to the persistence of habit-like drug seeking behavior (Hyman et al., 2006). Furthermore, nicotine- and stimulant-induced degeneration of the fasciculus retroflexus has been proposed to be related to relapse in chronic drug users (Ellison, 2002). The modulation of the dopaminergic pathway by the habenulo-interpeduncular pathway, as demonstrated in this study, may enhance our understanding of interrelated neural systems involved in addictive behavior.

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